Chapter 8: Phenotyping of Host-Pathogen Interactions in Mice

8
Phenotyping of Host–Pathogen Interactions in Mice

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8.1
Introduction

Infectious diseases are still a major cause of morbidity and mortality worldwide. One of the major challenges in contemporary infection research is the development of suitable animal models to further advance our understanding of host–pathogen interactions. There is an urgent need to better understand (1) the strategies used by pathogens to evade the host immune response; and (2) the mechanisms that the host immune system employs to fight off infectious agents. This is a prerequisite for improving the current prevention and therapeutic strategies that are used in the battle against infectious diseases.

Susceptibility or resistance of the host to infection is determined by a complex interplay of environmental, host, and pathogen factors. In the past, our approaches to understanding infectious diseases focused mainly on the study of either individual environmental or pathogen related factors while the role of the host was limited to studies on pathogenesis and adaptive immune responses. A few years ago this changed and now more attention is directed towards analyzing the contribution of host genetics to the process of infection. In addition, our knowledge of the mechanisms of natural or innate immunity has been largely extended mainly due to genetic studies in model organisms [1–4]. The mouse as a genetically tractable mammal has played a pivotal role in defining new pathways and gene functions that are important for host defense. A crucial requirement for the genetic dissection of the host immune response to pathogens in mice is the development of robust and standardized phenotyping assays [5]. Without standardization and comprehensive phenotyping it is not possible to identify relationships between genes and phenotypes and thus to discover new gene functions.

After giving a brief summary of the history of approaches that have been used in mice to analyze host defenses we will shortly review how these studies helped to improve our understanding of the mechanisms of infection susceptibility in man. We will give a few examples of how mouse genetics has facilitated the identification of critical host proteins that are involved in immune defense. It is impossible to adequately review all of the work that has been carried out using different classes of pathogens in mice. Therefore, we will focus on mouse models of bacterial infec-

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tion, which are also under investigation in our laboratories and at our research center. For further reading on mouse models of viral and parasitic infection, we can recommend some of the excellent reviews that have been published recently [6–8].

8.2 Looking Back and Forward: History and State-of-the-Art of Mouse Infection Phenotyping and Studies of Genetic Infection Susceptibility

The influence of genetic factors on resistance and susceptibility to bacterial infection in the mouse were first analyzed systematically in the early work of Leslie Webster at the Rockefeller Institute for Medical Research in New York [9, 10]. In fact, back in the early 1930 s, he was the first to establish what is today called "baseline-data" for different inbred strains of mice. He started to develop a mouse model of human typhoid fever by infecting mice orally with *Bacillus enteritidis* [9], now known as *Salmonella enterica* Serovar Typhimurium (or sometimes as the shortened but incorrect form, *Salmonella typhimurium*). He standardized many critical parameters of his experiments and thereby pioneered the reproducible investigation of host responses in animal models under controlled conditions (e. g. temperature, diet, age, weight and sex of mice [10]). Webster defined new standards of mice handling and housing by paying careful attention to the monitoring and maintenance of hygiene conditions in his mouse colonies [9]. He analyzed the influence of different routes of *Salmonella* inoculation (e. g. oral, intravenous, and subcutaneous application of the pathogen) on the outcome of infection and thoroughly investigated parameters such as dose of infection and kinetics of survival over time [10]. His studies enabled him to set up a mouse typhoid infection model by selective inbreeding of high-mortality and low-mortality lines of mice. He started to explore the heritability of infection susceptibility and demonstrated that "genetic factors" segregated in the backcross progenies of his newly established salmonella-resistant and -sensitive mouse strains. He concluded for the first time that "inherited components of resistance affect the response of the host to infection" [9]. This conclusion was possible because Webster tried to minimize the effects of interfering environmental factors. In particular, he worked with mice that were free from pre-existing infections with *Salmonella* or other pathogens, conditions to which not many of his colleagues paid attention to at that time. Today, a "specific pathogen-free (SPF)" standard in mouse breeding facilities is still an important requirement for any immunological study. Below we will discuss the impact that hygiene conditions can have on the outcome of infection challenge experiments and the standards necessary for the analysis of immune responses in mice under infection challenge conditions (see Sections 8.4 and 8.5.4).

Another well-established model is murine listeriosis. It has been studied for the past four decades to examine basic aspects of innate and acquired cellular immunity. Listeriosis is caused by *Listeria monocytogenes* which has emerged as a remarkably tractable pathogen with which to investigate basic aspects of intracellular pathogenesis. Infection challenge experiments with *L. monocytogenes* have been proven to be one of the most successful experimental models in history for defining

mechanisms that underlie immunity and host defense to infectious diseases. Fundamental concepts in immunology, such as macrophage activation [11, 12], the role of $CD4^+$- and $CD8^+$-T cells [13], major histocompatibility (MHC) restriction [14], adoptive transfer of T cell-mediated immunity [15], and the function of cytokines (for a detailed review see [16]), were derived from or further explored in this model.

L. monocytogenes is a facultative intracellular, Gram-positive bacterium that causes sepsis and meningitis in immunocompromised patients and devastating maternal/fetal infection in pregnant women. In 1962, George Mackaness described the first experimental model of listeriosis in mice [11]. Mackaness was interested in the immunological basis of non-humoral and acquired resistance to infection and investigated the pathogenesis of listeriosis in mice. He established assays for the examination of macrophage responses to *L. monocytogenes* in mice by isolating the cells from the peritoneal cavity and infecting them with *Listeria in vitro*. His readout assays for the bactericidal activity of the macrophages were quite simple and effective; he counted lytic plaques in cellular monolayers and enumerated ingested bacteria in macrophages by microscopy after staining with May–Grunwald–Giemsa [11]. More importantly, he developed a delayed-type hypersensitivity assay for *Listeria* by injecting a sterile filtrate of *L. monocytogenes* into the hind footpads of previously sub-lethal infected mice and afterwards examined the swelling of the footpads over time. This technique enabled him to monitor the response of mice to re-infection with *L. monocytogenes* [11] and it was one of the crucial methods that allowed him to define the general basis of cellular immunity to pathogens. By combining the delayed-type hypersensitivity assay with the transfer of lymphoid spleen cells from *Listeria*-infected mice into naïve mice, he was able to demonstrate that the transferred cells conferred protection against subsequent *L. monocytogenes* infection [15]. This was the starting point for the investigation of mechanisms of cellular immunity.

The genetic control of immune responses to infection with *L. monocytogenes* in different inbred strains of mice soon became a subject of intensive studies [17–19]. C57BL/6J, C57BL/10Sn, and B10.A mice were reported to be resistant to *L. monocytogenes* infection, whereas A/J, BALB/c, CBA/J, C3H/HeJ, DBA/1J and DBA/2J mice have been shown to be sensitive to infection with this pathogen [17, 18]. In Emil Skamene's laboratory genetic linkage studies using the AXB and BXA recombinant inbred (RI) lines of mice that were established from A/J and C57BL/6J parental strains of mice led to the mapping of two loci involved in the control of bacterial growth in the group of the susceptible RI strains [20]. Co-segregation with alleles at the *hemolytic complement* (*Hc*) locus on mouse chromosome 2 suggested early on that a deficiency in complement factor C5 was responsible for the susceptibility of the A/J parental strain. This was further supported by the observation that A/J mice were protected from *Listeria* infection when they were reconstituted with C5-rich serum from the $C5^+$ B10.D2/nSN strain [20]. More recently, the genetic susceptibility to *L. monocytogenes* infection in C57BL/6ByJ and BALB/cByJ mice was further refined by the mapping of two loci controlling systemic infection to mouse chromosomes 5 and 13 [21].

Today, the use of RI strains has again become very popular in the study of the complex genetics of pathogen defense. A powerful new genetic tool will be developed within the next years that will probably allow a new roadmap of interac-

tions between genes and polygenic networks to be defined. The complex trait consortium will establish the 1K-Collaborative-Cross (1KCC) of RI strains. It is planned to generate about 1000 new RI strains of mice from eight different parental inbred strains that can be used for phenotyping and high-resolution trait mapping [22]. One of the main attractions of the 1KCC system is the possibility of combining traditional genetic methods with novel systems-biology approaches.

The identification of the *Lsh/Ity/Bcg* locus is an impressive early example of the positional cloning of a single host gene in the mouse that is responsible for the susceptibility of different inbred strains to taxonomically unrelated intracellular pathogens. This locus mediates resistance of mice to *Leishmania donavani* (*Lsh* locus), *Salmonella enterica* Serovar Typhimurium (*Ity* locus), and to a number of different Mycobacterial species such as *Mycobacterium bovis* BCG (*Bcg* locus). Genetic susceptibility to these pathogens is caused by a mutation in the *Slc11a1* gene (previously known as natural resistance-associated macrophage protein 1, Nramp1) [23], which has been suggested to be a pH-dependent divalent cation efflux pump at the phagosomal membrane of macrophages (for a review see [24]). In phagocytes the Slc11 a1 protein has been implicated in acidification and maturation processes of the phagosome, which are important for intracellular, bactericidal host defense.

Screening the responses of mice to bacterial products instead of using living pathogens can also reveal host factors involved in pathogen resistance. Variation in inflammatory responses in inbred strains of mice after challenge with purified lipopolysaccharide (LPS) had already been found 40 years ago (for review see [25]). LPS is an abundant glycolipid present in the outer membrane of Gram-negative bacteria, which can provoke generalized, inflammatory responses in the infected host. Hyporesponsiveness to LPS can render mice highly susceptible to Gram-negative infections with pathogens such as *Salmonella* and *Klebsiella* because macrophage activation is impaired. Defects in LPS signaling in C3H/HeJ and C57BL/10ScN mice were found to be under control of the *Lps* locus on mouse chromosome 4. This locus was identified as the *Toll-like receptor 4* gene and the positional cloning of *Lps* first demonstrated that mutations in this class of pathogen-recognition receptors can profoundly affect susceptibility to infectious agents [26, 27]. The Toll-like receptor (TLR) gene family now comprises 11 members in mice (*Tlr1-Tlr11*, [28]) and is one of the best-studied immune sensors for invading pathogens. The signaling pathways triggered after recognition of *pathogen-associated molecular patterns* (PAMPs), which are the evolutionary conserved products of microbial metabolism initiate innate immunity and help to strengthen adaptive immune responses. Several important Tlr-adaptor molecules and downstream Tlr-pathway regulator proteins were identified through *N*-ethyl-*N*-nitrosourea (ENU)-mutagenesis screens in mice [29] or via gene targeting approaches and the subsequent phenotyping of PAMP responses in *Tlr*-deficient mice (for reviews see [30, 31]). In addition, other PAMP recognition receptors such as the Cd36 molecule were linked with hypersusceptibility to Gram-positive pathogens (e. g. *Staphylococcus aureus*) using ENU mutagenesis approaches in the mouse [32]. Here, the laboratory of Bruce Beutler at the Scripps Institute in San Diego has played a pivotal role in establishing specialized ENU mutagenesis screens in mice which allow the systematic genetic investigation of the mouse immune and host defense system [29, 32, 33].

Within the last few years many valuable new techniques have been developed for phenotyping immune responses in infected mice. In particular, many new non-invasive techniques have been designed to image host defense responses in mice. These are very interesting developments because they have the potential for infection phenotyping of mice in high-throughput primary screens. Currently, the characterization of infection susceptibility is very laborious, time consuming, and usually requires many animals to monitor the kinetics of pathogen dissemination and to examine the organ pathology of the mice after infection. With new diagnostic tools in imaging this might change in the future. Such efforts need support because non-invasive imaging techniques will be crucial tools for experimental pharmacological research and antimicrobial drug development. Examples of modern imaging technologies are two-photon microscopy [34], magnetic resonance imaging (MRI) [35], and *in vivo* bioluminescence imaging of pathogen dissemination in the entire body of an infected mouse over time with sensitive charge-coupled device (CCD) cameras [36, 37]. Another interesting new imaging technique is the *in vivo* reporter enzyme assay which uses radiolabeled substrates that allow the *in situ* detection of pathogens with single-photon emission computed tomography (SPECT) [38].

A very important method for monitoring the induction and maintenance of T-cell responses to infectious agents is the tetramer staining technique which facilitates investigation of the function of T cells in recall infection experiments [39, 40]. Most T cells recognize peptides derived from pathogens that are bound to MHC molecules on the surface of target cells or antigen-presenting cells (APCs). This recognition is specific for both the MHC allele and the pathogen-derived peptide. Flow cytometry can be used to detect soluble peptide–MHC complexes attached to fluorochromes thus making it possible to identify antigen-specific T cells. However, due to the poor binding of monomeric peptide–MHC complexes to the T cell receptor, the use of multimers which are typically tetramers, is required. For staining, the purified MHC molecules are biotinylated and then added to fluorescently-labeled streptavidin complexes in solution. The tetramer staining technique was essential to study for example, the transition from primary effector T cell to memory T cell responses after *L. monocytogenes* infection [41] and for the characterization of $CD8^+$ T cell responses to this pathogen [42].

8.3
The Impact of Mouse Genetics on the Understanding of Human Infectious Diseases

Primary immunodeficiency diseases in humans consist of a group of more than 100 inherited clinical manifestations that can predispose individuals to different infectious diseases, allergy, autoimmunity and cancer [43]. Many of the identified genes that have been associated with abnormal or deficient immune responses in patients had been identified before using the mouse as a model system (see also Chapter 10 of this volume). Mutations in genes that lead to primary immunodeficiencies are often associated with recurrent infections in patients that are caused by very diverse microorganisms. A good example of such a generalized immunodeficiency is chronic granulomatous disease (CGD), which is characterized by frequent

infections with pathogens such as *Staphylococcus aureus*, *Aspergillus fumigatus*, *Salmonella* (non-typhoid serovars), *Serratia marcescens* and *Burkholderia cepacia* (for a review see [44]). CGD can be caused by inherited mutations in genes that encode the gp91-phox, p47-phox, p22-phox or p67-phox subunits of the NADPH-dependent phagocyte oxidase (for a review see OMIM database http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM and [44]). Murine models of CGD that lack functional *Cybb* ($gp91^{phox}$; [45]) or *Ncf1* ($gp47^{phox}$; [46]) alleles, recapitulate the immune defects that are observed in man and can be considered as geno- and phenocopies of the human disease.

Mutations and disease-associated polymorphisms in TLR-pathway genes or in the TLR-encoding genes that are themselves responsible for the predisposition of patients to infectious diseases, have also been identified. This was achieved through the extensive work that had previously been carried out in mice to unravel the underlying mechanisms of host defense. A dominant *TLR5* stop codon mutation that abolishes the recognition of bacterial flagellin could be linked to susceptibility to Legionnaires' disease. This was confirmed after a large outbreak of the disease at a flower show in the Netherlands in 1999 by the subsequent screening of affected individuals for sequence variants in the *TLR5* gene [47, 48]. *TLR5* was a candidate gene because the molecular and functional characterization of this receptor had previously been established using knockout mice [49]. Other examples of mutated human genes that are involved in TLR signaling are deficiencies in *IRAK4* which cause hyporesponsiveness to LPS and a predisposition to pyogenic bacterial infections [50, 51] and variations in the *TLR4* sequence that may be linked to the development of meningococcal sepsis in patients [52] and to resistance to Legionnaires' disease [53].

Mendelian susceptibility to mycobacterial diseases (MSMD) is a rare syndrome of severe infections caused by low-virulence mycobacteria and *Salmonella* in patients carrying mutations in five genes of the IL-12-IFN-γ-STAT1 signaling axis. Interestingly, these patients seem to be resistant to other pathogens, while knockout mice with deficiencies in the homologous genes are widely susceptible to many different microorganisms (for a detailed review see [54]).

Since the early studies of Emil Skamene on complement factor C5 deficiency in mice and their susceptibility to *Listeria* infections, it would seem that the complement-activation product C5 a in particular, may be associated with the development of sepsis in humans (see the recent detailed review by Peter Ward [55]).

The mouse has been instrumental in accelerating the advances in immunological research which have been made in recent decades [56, 57]. However, it must be remembered that discoveries made using mice do not necessarily lead to corresponding insights in humans. Different selection pressures (many caused by parasites and pathogens) over the last 65 million years of evolution have left their imprints on the human and rodent genomes. These are also responsible for the differences between mouse and human immunology [58]. Nevertheless, mice will remain the prime experimental model of choice for immunological research and defined differences in host defense genes between both organisms might be tackled in the future by directed transgenic approaches (e. g. humanization of mice through "knock in" procedures or replacement with human genes).

8.4
Phenotyping at the GBF-Mouse Infection Challenge Platform (ICP)

At our research institution we are working primarily with three bacterial pathogens to characterize the innate and adaptive immune responses of mice. The infection experiments are performed under controlled "SPF" conditions (see also Section 8.5.4). Mice are housed in individually ventilated cages (IVCs) and are handled only in protected areas (under laminar flow hoods). All material, which is brought into the animal infection unit, is either autoclaved or sterilized with H_2O_2. An extensive sentinel program is used to screen the unit every 3 months for the presence of unwanted microorganisms. New mouse strains from external sources are imported into the facility via embryo transfer. In addition, the "altered Schaedler flora (ASF)" is used to colonize the gastrointestinal tracts of transferred mice with a defined microflora [59]. To accomplish this, germ-free foster mice that have previously been colonized with the ASF are used for embryo transfers.

We use *Streptococcus pyogenes* as an extracellular, Gram-positive pathogen to investigate cellular mechanisms and molecules that are linked to the induction of bacteremia and sepsis [60]. Within the last few years, this infection model has proven very useful in the elucidation of immune mechanisms underlying disease susceptibility to streptococcal-induced sepsis in the mouse (for more information see [61]).

To investigate mucosal immune responses to pathogenic Gram-negative bacteria in the gut we established a low-virulence infection model with *Yersinia enterocolitica*. Here, the strain *Y. enterocolitica* E40 serotype O:9 is employed to specifically analyze responses to local infection in the intestine and Peyer's patches. Mice are infected with *Y. enterocolitica* by the natural route (oral) and immune responses are monitored at 3, 9, and 21 days after infection. The model is very informative but also quite labor intensive because it involves histology as one of the major out-read-systems for infection susceptibility and resistance (Frischmann, U. and Müller, W. in preparation). Its main advantage over other mouse infection models is that the presence of infectious lesions and the influx of various immune effector cells after infection can be monitored effectively at very high resolution (e. g. the cellular level). To test new mutant lines of mice for general defects in host defense we use the intracellular pathogen *Listeria monocytogenes*, which has been mentioned above. The screening protocols used for this pathogen will be listed below. Additional infection protocols for the other ICP-pathogens, *S. pyogenes* and *Y. enterocolitica* can be accessed at the EUMORPHIA website (http://www.eumorphia.org). Within the last 3 years we have established standardized operation procedures (SOPs) for infection phenotyping of mice in the framework of EUMORPHIA that can be used for reference. In addition to the SOPs for mouse infection challenge with *S. pyogenes*, *L. monocytogenes*, and *Y. enterocolitica*, other associated documents and primary-extended screens for macrophages (e. g. PAMP responses) can be found on this website.

8.4.1 Sreening Protocols

8.4.1.1 Infection with Listeria monocytogenes

The mouse model of *L. monocytogenes* infection has been used extensively to investigate immune responses to bacterial infection [16]. The response of the mouse to *L. monocytogenes* infection can be divided into an early non-specific inflammatory response, which is initiated after recognition of the pathogen by the innate immune system, and a more delayed specific immune response which is mediated by the adaptive immune system (Fig. 8.1). *L. monocytogenes* can be administered through different routes of infection. Inoculation of *L. monocytogenes* into mice can be carried out orally, intraperitoneally (i. p.), subcutaneously (s.q.) or intravenously (i. v.). However, although infecting mice orally is the natural route of infection for *L. monocytogenes*, high doses of the bacterium are required for the successful establishment of a systemic infection. This is thought to be due to the species-specific interaction of the *Listeria* protein internalin A on the surface of the bacterium with its host cell receptor E-cadherin [62]. The interaction of both proteins mediates the adhesion and invasion of *Listeria* into the epithelial cells of the intestine. Humans and mice have a different amino-acid residue at a critical site in the E-cadherin protein [63]. It has been suggested that this difference in E-cadherin is responsible for the host-specific tropism of *Listeria* in the gut. In our laboratory we use the intravenous route of infection. This inoculation method is highly reproducible and results in a rapid systemic infection. Different strains of *L. monocytogenes* can vary in virulence. For our infection protocols we use the *L. monocytogenes* strain EGD, which is commonly used for phenotyping and has an intermediate to high virulence in mice when compared to other available strains of *Listeria* [64]. The advantage of this strain is that a European consortium has sequenced its genome [65] and many isogenic *L. monocytogenes* mutants are available in this genetic background. This allows the use of bacterial mutants, which are attenuated in virulence due to the deficiency of critical virulence factors. Therefore, these *Listeria* mutants can be used to phenotype the host response of very susceptible mouse mutant strains that would otherwise immediately succumb to the infection (for instance, *interferon-γ* gene knockout mice). In general infection doses ranging from 10^2 (sublethal) to 10^5 colony forming units (cfu) are used, depending on the LD_{50} of the mouse strain under investigation. As already mentioned, the most commonly used inbred mouse strains differ significantly in their susceptibility to *Listeria* infection [17]. Therefore, it is advisable to test the LD_{50} of a particular mouse mutant strain before starting extensive experiments, especially when the mouse strain under investigation is maintained on a mixed genetic background. Together with the laboratory of Dirk Busch at the German Mouse Clinic (see also Chapter 10 in this volume) we have recently established extensive baseline data sets for the C57BL/6J, C3H/HeN, BALB/c and CBA/J inbred mouse strains that can be used for reference [66]. In the course of this project we made the interesting observation that infection of mice with *L. monocytogenes* is associated with a sex-dependent susceptibility pattern. Independent of the genetic background, female mice are in general more susceptible to the infection than male mice [66] and this should also be taken into account

when undertaking infection challenge experiments with this pathogen. Following i. v. infection of mice, *L. monocytogenes* is first confronted by macrophages and neutrophils in the spleen and the liver. Within the next 6–12 h of infection 60 to 90 % of the bacteria are killed and the organism can no longer be detected in the peripheral blood of immunocompetent mice. The early pro-inflammatory response in the liver is initiated by the interaction of the pathogen or its products with pattern recognition receptors (e. g. Tlr2 and Tlr5) on the surface of Kuppfer cells [67], which are the macrophages of the liver. The interaction of the bacterial PAMPs with TLRs induces signal transduction pathways that lead to the activation of transcription factors (e. g. NF-κB), which in turn promotes the production and release of pro-inflammatory cytokines involved in innate host defense mechanisms (e. g. IL-1, IL-6 and TNF-α). These events promote the accumulation of neutrophils at the local infection sites. These immune effector cells are the principal microbicidal population in the liver during the first 24 to 48 h of *Listeria* infection [68]. They eradicate most of the bacteria and produce additional cytokines and chemokines that attract and further stimulate additional immune effector cells, such as monocytes, granulocytes, natural killer (NK) cells and T cells. The cytokine IFN-γ which is mainly produced by NK cells and T cells of the T-helper cell type-1 type (Th1), in turn activates macrophages that phagocytose and kill the pathogen inside the infected tissue. These responses substantially reduce the infectious burden of the animal and are essential for the survival of the infected mice. If these initial host reactions fail, the mice will eventually die within the first 2–3 days after infection. In these cases, histopathological analysis will reveal necrotic, multifocal granuloma in the liver and spleen and necrotic lesions caused by multiplying *Listeria* in the bone marrow (Fig. 8.2). During the normal infection process in an immunocompetent host, a fraction of the *L. monocytogenes* taken up by the liver escapes the antimicrobial activity of neutrophils and macrophages and will spread to hepatocytes, in which the bacteria will further replicate intracellularly. In immunocompetent mice, T cells will finally mediate the clearance of the pathogen and will eventually provide long-term immunity. This specific immune response to the pathogen occurs 5 to 7 days after infection and is mainly mediated by cytolytic $CD8^+$ T cells (for a review see [69]). If these T cell-mediated adaptive immune responses fail the mice will die from the infection 7 to 10 days after inoculation. To test T cell immunity specifically to *Listeria monocytogenes* we recommend the recall infection experiments described in Chapter 10 of this volume.

When live *Listeria* are used for infection challenge experiments in mice, it should be remembered that the bacteria are pathogenic organisms that can cause severe, sometimes lethal infections in immunocompromised patients or in individuals receiving immunosuppressive drugs. Therefore, all work carried out using *L. monocytogenes* must be performed under Biosafety Level 2 (BL-2) conditions. Investigators, who decide to work with this pathogen, should consult their local Biological Safety Department for guidance and approval prior to planning any experiments. If standard precautions are observed and followed, work with this microorganism can be carried out quite safely. The fact that detailed microbial and immunological characterization has been carried out on *L. monocytogenes*, makes it one of the safest and most popular pathogens to be used in experimental immunology worldwide.

Early innate immune response
Macrophages
PMNs
NK cells
IFN-γ
TNF-α
CCL-2
IL-12
IL-6
IL-8
Recruitment
CD4-T cells
CD8-T cells
PMNs
Monocytes
Delayed specific immune response
CD8-T cells
Granzymes
Perforin
intracellularly infected
Hepatocytes
FasL
Fas
Fas
FasL
FasL
Fas
CD4-T cells
IFN-γ
IL-12
IL-2
IFN-γ
IL-12
IL-2
Hepatocyte
apoptotic
Hepatocyte
PMN
CD4-T cells
Macrophage

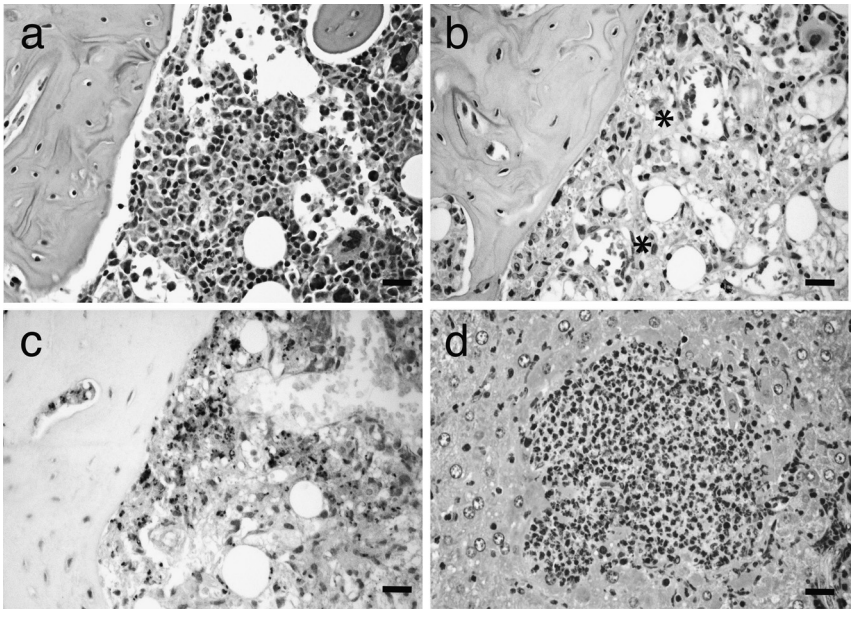

Fig. 8.2 Histopathological analysis of *Listeria monocytogenes* infections in mice. (a) Transverse section of the bone marrow from a CBA/J male 3 days after infections (*L. monocytogenes* EGD, 1×10^4 cfu , i. v.). (b) Transverse bone marrow section of a CBA/J female 3 days after infection with the same infection dose as in (a); note in (b) in comparison to (a) the acute and focal necrosis in the epiphysis of the bone marrow (indicated by stars). Female mice are more susceptible to *L. monocytogenes* infection than male mice. Sections in (a) and (b) were stained with hematoxylin and eosin. (c) Bone marrow section from the female shown in (b) stained with an anti-*Listeria* antibody. Bacteria are multiplying in the bone marrow at the sites of cellular necrosis (brown color). (d) Transverse liver section of a CBA/J female 1 day after i. v. infection with 1×10^4 cfu *L. monocytogenes* EGD. Granulomatous hepatitis with central necrosis and infiltration of neutrophilic granulocytes are visible. Scale bars = 12.5 µm.

◁ **Fig. 8.1** Early innate and delayed specific host immune responses to *Listeria monocytogenes* in the liver. Important effector cells involved in the immune response and released cytokines and chemokines are shown (PMNs, polymorphonuclear neutrophils; NK, natural killer cells). Cytolytic CD8-T cells which are involved in the specific immune response to *L. monocytogenes* kill infected hepatocytes through induction of apoptosis via the FAS ligand (FasL)/FAS receptor pathway or the perforin and granzyme pathway.

8.5
Practical Guidelines

8.5.1
Growing Log-phase Cultures of *Listeria monocytogenes* EGD for Mouse Infection

Reagents Phosphate buffered saline (PBS) pH 7.0, Brain Heart Infusion Medium (BHI from Becton and Dickinson, MD, USA; dissolve 37 g BHI powder in 1 l H_2O, autoclave and store at 4 °C), BHI plates (add 15 g agar to 1000 ml BHI medium, autoclave, pour plates and store at 4 °C for a maximum of 4 weeks), 0.4 % Trypan blue (dilute 400 mg Trypan blue (Sigma) in 100 ml H_2O, sterile filter (0.22 µm) and store at room temperature.

Equipment Laboratory centrifuge (with temperature control), incubator set at 37 °C, shaking incubator (37 °C, 110 r.p.m.), photometer set at a wavelength of 600 nm, microscope (upright, no inverse) with 10 × ocular and 20 × (brightfield) and 40 × (phase contrast) objectives and phase contrast condenser, Thoma chamber (normal chamber depth, 0.1 mm), laminar-flow bench (BL2 level).

Procedure

1. Day 1: plate *Listeria* from a fresh glycerol stock on a pre-warmed (37 °C) BHI plate. Let bacteria grow for 24 h at 37 °C.
2. Day 2: inoculate a single colony from the BHI plate into 6 ml of BHI medium in a 14-ml snap cap tube. Let bacteria grow overnight at 37 °C in a shaking incubator (110 r.p.m.).
3. Day 3: measure OD_{600} of the overnight culture, OD should be around 1.0. Dilute overnight culture 1 : 10 in BHI medium (3 ml bacteria in 27 ml BHI) in an Erlenmeyer flask. Measure OD_{600} of the dilution. Let the bacteria grow at 37 °C in a shaking incubator (110 r.p.m.).
4. After 2 h of growth start to measure OD_{600} in 5–10-min intervals until an OD_{600} of 0.5 is reached. Centrifuge bacteria for 5 min at 1600 *g*, 4 °C. Pour off the supernatant and add 10 ml of ice-cold PBS. Re-suspend the pellet carefully using a pipette. Centrifuge for 5 min at 1600 *g*, 4 °C. Pour off the supernatant and re-suspend the pellet in 25 ml of ice-cold PBS. Dilute 100 µl of bacteria in 900 µl of 0.4 % Trypan blue. Count the cells under a microscope using a Thoma chamber. Dilute *Listeria* to a concentration of 1×10^6 cfu/ml in PBS.
5. Prepare 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} dilutions of the *Listeria* using PBS. Plate 100 µl of the 10^{-2}, 10^{-3} and 10^{-4} dilution on BHI plates, each on three separate plates. Incubate overnight at 37 °C. Count the colonies on the following day and calculate the concentration of *Listeria* inoculum (cfu) used in the experiment.

8.5.2
Infection of Mice with *Listeria monocytogenes* EGD

Reagents Phosphate buffered saline (PBS), pH 7.0.

Equipment Infrared light (e. g. Philips Infrared PAR-38, 150 W), mouse strainer (e. g. Plas-Labs, #551-BSSR), syringe with 29 G needle (e. g. B.Braun Omnican®-F), Laminar-flow bench (BL2 level).

Procedure

1. Use the *L. monocytogenes* inoculum prepared from the protocol above. Dilute bacteria to 1.5×10^5 cfu/ml in PBS. An injection of 100 µl is equivalent to an infection dose of 1.5×10^4 cfu per mouse which is the LD_{50} for female C57BL/6J mice. If the mouse strain of interest is more susceptible or resistant in an initial experiment, repeat the infection procedure using a lower or higher dose respectively.
2. In a laminar flow hood open the animal cage under infrared light for at least 2 min. The distance between the top of the cage and the bulb should be approximately 20 cm.
3. Pre-warm mice individually by placing each mouse on top of the cage under the bulb for 15–30 s. Place the mouse in a mouse restraining device.
4. Inoculate 100 µl diluted *L. monocytogenes* into the lateral tail vein (veins are visible on both sides of a pre-warmed mouse tail). Start injecting near the tip of the tail.
5. Survival rates can then be determined with a daily health check of the infected animals for a period of 14 days (for more detailed instructions see "SOP Health monitoring of mice in infection experiments", Workpackage 6 accessible at http://www.eumorphia.org, or upon request). Animals that survive for 14 days are ranked as "resistant".

8.5.3 Quantification of Bacterial Growth in Spleen and Liver after *L. monocytogenes* Infection

Reagents Phosphate buffered saline (PBS) pH 7.0, Brain Heart Infusion Medium (BHI from Becton and Dickinson, MD, USA; dissolve 37 g BHI powder in 1 l H_2O, autoclave and store at 4 °C), BHI plates (add 15 g agar to 1000 ml BHI medium, autoclave, pour plates and store at 4 °C for a maximum of 4 weeks).

Equipment Laboratory centrifuge (with temperature control), incubator set at 37 °C, automated tissue homogenizer, Laminar-flow bench (BL2-level).

Procedure

1. Sacrifice mice by cervical dislocation on day 2 or 3 after infection (depending on the survival curve of the previous experiment; use time points prior to the deaths of the first mice).
2. Aseptically remove liver and spleen. Weigh all organs and put in a 14-ml Snap cap tube containing 5 ml pre-cooled (4 °C) PBS. Store all organs on ice until all mice have been sacrificed and dissected.
3. Homogenize tissues for 30 s using an automatic homogenizer. Clean out homogenizer between two samples by squirreling with PBS (2×5 s), 70 % ethanol (1×5 s) and PBS (1×5 s).

4. Prepare dilutions of tissue homogenates (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions) using PBS and snap cap tubes. Plate dilutions onto BHI plates in triplicate. Store dilutions at 4 °C for further dilutions (if necessary).
5. Next day, count colonies and calculate total cfu/organ and cfu/mg organ. If the 10^{-5}-dilution plates are still overgrown, prepare further dilutions (10^{-6}, 10^{-7}, and 10^{-8}) and plate again in triplicate.

8.5.4 Troubleshooting

Critical parameters for infection challenge experiments and immunological phenotyping are as follows:

- "Specific pathogen free" (SPF) housing conditions for mice. Background infections can severely alter the immune status of mice and can therefore have a deep impact on the outcome of infection experiments [70]. In our mouse facility we follow the recommendations of the Federation of European Laboratory Animal Science Association (FELASA) to maintain standardized hygiene conditions [71].
- Standardization of experiments and inclusion of internal controls. For infection experiments age- and sex-matched animals should be used. To control for inoculum quality and technical effectiveness of infection we use mice from known susceptible inbred strains as internal controls. These are infected together with the experimental group of mice and the results in this control group should match the previously recorded baseline data for this particular strain.
- It is advisable to use standardized microbiology laboratory practice to exclude contamination of bacterial stocks, inoculum, and experimental samples. Protective clothing should be worn when handling and manipulating bacteria, samples and animals and all procedures should be carried out in a laminar-flow hood.

8.6 Outlook

The identification and characterization of infectious disease loci in mice cannot be successfully accomplished without *in vivo* infection experiments combined with sophisticated and detailed phenotypic analysis. To further investigate the molecular and cellular mechanisms of host–pathogen interactions in the future there is a need to extend the currently available genetic toolbox for the mouse. We need to establish new transgenic mouse lines that allow a spatial and temporal depletion of critical immune effector cells during the host defense response. For example, conditional expression of cholera toxin could be used to specifically deplete the immune system of cells of the myeloid or lymphoid cell lineage. This would help to characterize the contributions of specialized or activated cells (such as different types of macrophages) to the host defense response.

We also need to further miniaturize the readout assays and systems that are used to monitor the host response in a non-invasive manner. New advances in

proteomics technology should be combined with immunology. The new emerging field of "immunoproteomics" is very promising in this respect [72, 73]. New techniques should help to identify novel surrogate markers of immune responses that can subsequently be used to establish new high-throughput diagnostic tools (e. g. antibody arrays for the detection of antigen-specific immunoglobulins or new acute-phase serum proteins). New imaging technologies will definitely have a deep impact on the phenotyping of infection responses. They will elucidate the complexity and dynamics of infection processes *in vivo* without disturbing or interfering with the integrity of the different host immune compartments that are involved in defense mechanisms against pathogens. Some examples of emerging new imaging technologies have already been mentioned in this chapter and in Chapter 10.

The challenge of the future is to identify and understand the diverse intersection points where pathogen virulence factors interfere with host defense and metabolism. Systems biology approaches combined with new quantitative readout assays for "immunophenotypes" might be the key to understanding regulatory and signaling networks that are most critical for defense against pathogens and drug development.

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